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ANNUAL REPORT

Daniel G. Baden and Thomas J. Mende

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Fort Detrick, Frederick, Maryland 21701-5012

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The polyether lipid-soluble toxins isolated from the marine dinoflagellate Ptychodiscus brevis (formerly Gymnodinium breve) bind to a unique site, Site V associated with voltage-dependent sodium channels in rat brain synaptosomes. Using tritiated PbTx-3 as a specific probe for binding at Site V, a K_d of 2.9 nM and a B_{max} of 6.8 pMol/mg synaptosomal protein has been determined. Binding equilibria and displacement by unlabeled PbTx-3 occur in a comparable concentration range to that of saxitoxin (Site I). Labeled toxin can be displaced in a competitive manner by any of the other 5 naturally-occurring toxins; the quantitative displacement ability of each appears to reflect individual potency in fish bioassay. Preliminary K_i calculations have been made for four of the toxins. Two separate photoaffinity probes have been synthesized and have been covalently-linked to PbTx-3. Each complete photoaffinity-toxin conjugate competitively displaces tritiated PbTx-3 from it specific site, with ED_{50}s in the 20-50 nM ranges.

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Summary

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Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) haved adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, revised 1985).

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II. Statement of the Problem

The research described is aimed at characterization of the binding site for Ptychodiscus brevis neurotoxin PbTx-3 (formerly known as T17) in nerve tissue, specifically in rat brain synaptosomes. To achieve this objective, we have:

(1) prepared synaptosomes according to established techniques;

(2) determined the degree of toxin PbTx-3 binding to synaptosomes using ^{3}H-labeled PbTx-3, and have determined apparent K_D and B_{max};

(3) determined the degree of displacement of labeled PbTx-3 by other natural and synthetic derivative of brevetoxins;

(4) determined the degree of competition between underivatized brevetoxin and synthesized photoaffinity derivatives of brevetoxin PbTx-3 for the specific site in synaptosomes;

(5) developed a radiosynthetic technique for derivatizing PbTx-1 (formerly known as brevetoxin-A), the most potent brevetoxin, to a high specific activity probe.

III. Background

A. History

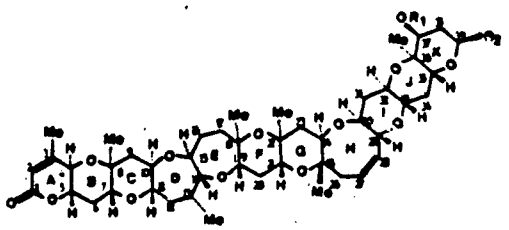
In last year's Annual Progress Report (1), we outlined previous work on the brevetoxins PbTx-2 and PbTx-3 , work that had been conducted over the past 13 years. We now routinely purify to homogeneity six brevetoxins, based on two structural backbones (Figure 1) (2). All toxins produced by P. brevis are ichthyotoxins, and in fact most investigators utilize fish bioassay to precisely identify potent fractions during purification. This complex of six toxins, namely PbTx-1,-2,-3,-5,-6, and -7, in composite are responsible for in situ fish kills during red tides in the Gulf of Mexico (3). In addition, these polyether materials are acutely potent in swiss white mice (4,5), as bronchoconstrictors (6), in in vitro phrenic nerve hemidiaphragm preparations (7), and in crayfish and squid giant axon (8). In varying degrees, they also elicit increased sodium ion influx in brain synaptosomes (9), and induce increased release of acetylcholine and decreased choline uptake in neuromuscular junction (3). Dose-response curves for each effect enumerated above were in the nM to pM concentration ranges. In all in vitro systems, each described effect was reversible by washing with fresh toxin-free bathing medium.

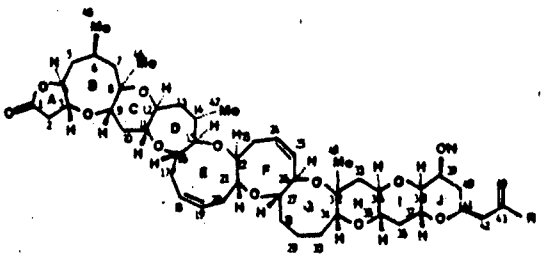
Squid giant axon and crayfish axon bundle experiments were particularly important in our initial work (8), for these gave us our first indication that brevetoxins interacted with specific sites of the axons. With a tetrodotoxin-sensitive mechanism of action, brevetoxins induced a sodium ion-sensitive dose-dependent depolarization

(30 mV maximum depolarization, linear dose-response between 0.2-100 nM PbTx-3). This data led us to postulate a specific binding site for the brevetoxins located on, or proximal to, the voltage-sensitive sodium channel (9).

B. Toxins

Since the identification of P. brevis as the progenitor of Florida red tide toxins, the potent entities have been know by several different names--most of which are important only for historical reasons. The name brevetoxins seems to have been adopted, but a widely used notation series has not been adopted. Following conventional notation for natural toxins (which is currently under review by several National and International working groups), we have adopted the notation PbTx-# for Ptychodiscus brevis Toxin 1-8 (Table 1). Additional toxins, if discovered, would continue with PbTx-9 etc. It is expected that, like the PbTx-2 backbone (Type 1), both epoxides and O-acetates of PbTx-1 (Type 2) will ultimately be discovered. The mass amounts of these materials is expected to be approximately as indicated in Table 2.





	R_1	R_2
PbTx-2	H	$CH_2C(=CH_2)CHO$
PbTx-3	H	$CH_2C(=CH_2)CH_2OH$
PbTx-5	Ac	$CH_2C(=CH_2)CHO$
PbTx-6	H	$CH_2C(=CH_2)CHO$ (27,28 epoxide)
PbTx-8	H	CH_2COCH_2Cl

	R
PbTx-1	CHO
PbTx-7	CH_2OH

No structural information available on PbTx-4

Figure 1. Structures of the Brevetoxins.

TABLE 1. NOMENCLATURE FOR THE BREVETOXINS*

Notation	Synonyms	Reference
PbTx-1	Brevetoxin-A	10,11
	GB-1	11,12
PbTx-2	Brevetoxin-B	13
	GB-2	11
	T34	4
PbTx-3	GB-3	11
	T17	5
PbTx-4	GB-4	11,14
PbTx-5	GB-5	11
PbTx-6	GB-6	11
PbTx-7	GB-7	11
PbTx-8	Brevetoxin-C	15

*refer to Figure 1 for structure details

TABLE 2. TOXIN YIELDS FROM CULTURES
TYPE 1 VERSUS TYPE 2 TOXINS

Type 1 Notation	Type 1 Yield (pg/cell)	Type 2 Notation	Type 2 Yield (pg/cell)	Note
PbTx-2	8.7	PbTx-1	1.7	(1)
PbTx-3	0.42	PbTx-7	0.026	(2)
PbTx-5	0.062	PbTx-9*	0.013	(3)
PbTx-6	0.037	PbTx-10*	0.008	(4)

*Toxins underlined have not been demonstrated in cultures. By analogy with Type 1 toxins, they are proposed to exist, and the yields given are in proportional amounts to Type 1 analogies. Notes: (1) alpha-beta unstaurated aldehyde; (2) alpha-beta unsaturated primary alcohol; (3) O-acetate; (4) epoxide.

C. Molecular Pharmacology

Initial binding experiments performed by others were indirect in nature, with respect to brevetoxin binding; i.e. unlabeled brevetoxin was used as potential competitor for other toxins known to bind to specific sites associated with voltage-sensitive sodium channels. Using this type of protocol, it was illustrated that PbTx-1 did not displace toxins which bind specifically at sites 1-3 located on, or proximal to, the channel (16). That brevetoxin did not interact with site 4 was presented (17).

By 1982, we were routinely producing tritiated PbTx-3 at greater than 10 Ci/mmole by reductive tritiation of PbTx-2 using sodium borotritiide (18). This probe was originally synthesized for radioimmunoassays which were being developed for public health use. We soon realized, in addition, that the probe would be invaluable in the measurement of specific binding of toxin to pharmacological site of action.

In vivo, binding of brevetoxin to site 5 of voltage-sensitive sodium channels is believed to be the pharmacologically-significant event in the onset of intoxication (9,17). Using tritiated brevetoxin PbTx-3 (C-42 tritium covalent label) as the specific probe, binding was determined at 4°C in rat brain synaptosomes using a rapid centrifugation technique (9). Rosenthal analysis yields a K_D of 2.9 nM and a B_{max} of 6.8 pmol of toxin/mg of protein (Fig 2).

We have shown that labeled probe can be displaced by unlabeled PbTx-3, PbTx-2, or synthetic PbTx-3 (reduced PbTx-2) but not by a nontoxic, synthetic oxidized derivative of PbTx-2 (9). Competition experiments using unlabeled natural toxin probes specific for sites 1-4 of the voltage dependent sodium channel illustrated that indeed the tritiated brevetoxin binds to a previously-undescribed site (9).

Based on the availability of additional naturally-occurring and derivative brevetoxins, we began a quantitative study of brevetoxin binding in this assay. The data we will present below illustrates that specific binding assays using sodium channel receptors reflect potency of the individual toxins in fish bioassays. In addition, we propose research directions for next year, including radiosynthesis of tritiated PbTx-7 (reduced PbTx-1) for comparison of specific binding characteristics of the two backbone structures presented in figure 1.

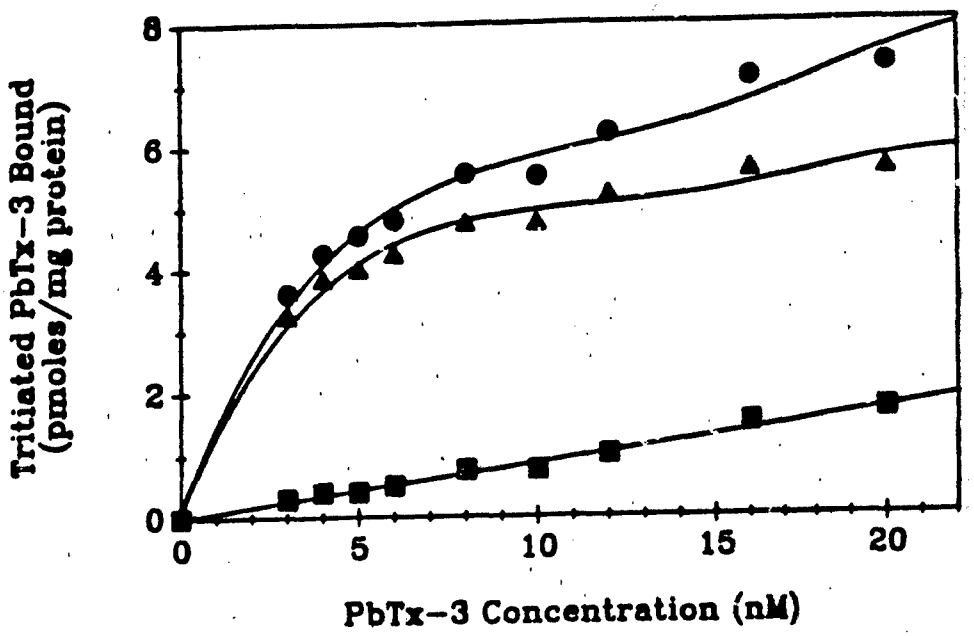


Figure 2. Concentration dependence of Tritiated PbTx-3 Binding to Rat Brain Synaptosomes. (●) Total binding, (▲) specific binding, (■) nonspecific binding [in presence of 10 uM unlabeled PbTx-3]. 4°C, 1 hour incubation.

IV. Technical Approach

A. Dinoflagellate Culture

Unialgal cultures of Ptychodiscus brevis were grown in the laboratory in 10 liter batches in glass carboys. Carboys were kept in continuous light at 4000 lux and 24°C in plexiglass constant temperature baths. Typically, 90 liters of culture are harvested each week, 650 liters of culture being grown continuously. Cultures at peak density, if not extracted, were diluted 50/50 with autoclaved NH-15 medium (19). Twenty-one days after inoculation, cultures reach maximum density. Cultures were harvested in either mid-logarithmic phase or in stationary phase, for reasons described in V. A. Toxins.

B. Toxin Purification

Toxin was extracted from whole 10 liter cultures by the addition of 1 liter of chloroform. Cells were disrupted and cultures mixed by use of a vibrating liquid homogenizer. Following flash-evaporation of the chloroform fraction, the residue was dissolved in 90% aqueous methanol and was extracted three times with equal portions of petroleum ether to remove nontoxic pigments. The residue which remained after evaporation of the methanol fraction was extracted with acetone and non-toxic insoluble materials were removed by centrifugation. The semipurified toxins were next subjected to three silica gel chromatographic procedures. The first silica gel step, a flash column, was performed using $CHCl_3$/methanol/acetic acid (100/10/1) as developing solvent. Two column volumes of solvent were passed through the column, collecting all eluent for flash-evaporation. This step is necessary prior to thin-layer chromatography.

A preparative thin-layer chromatography step using silica gel plates (1000 u thickness) utilizes acetone/petroleum ether (30/70) as solvent and resolved two toxic fractions (each of which is composed of multiple individual toxins)(R_f= 0.17 and 0.34). A second thin-layer chromatography step (500 u thickness) utilizes ethyl acetate/petroleum ether (50/50) for R_f= 0.34 material; a similar step utilizing a solvent system of 70/30 ethyl acetate/petroleum ether. The individual toxins can be visualized under short wave ultraviolet light. Toxins were eluted from the silica gel using acetone or methanol. TLC purified toxins were subjected to C-18 reverse phase high pressure liquid chromatography (1.4 mL/min, 85% methanol/15% water, isocratic, uv detection at 215 nm). The entire purification procedure requires two days; the first day progresses through both thin-layer steps (a substantial increase in efficiency over that outlined in year 1) and the second day is required for HPLC.

C. Synaptosome Binding Assay

Biological Preparation. Synaptosomes were prepared fresh daily from rat brain using the techniques described by Dodd et al. (20). Synaptosome integrity was evaluated using electron microscopy, or by ^{22}Na influx experiments. To prepare lysed synaptosomal fragments, the synaptosomal pellet was resuspended in 5 mM sodium phosphate (pH 7.4) and incubated with occasional stirring for 30 min in an ice bath. Protein was measured on resuspended intact synaptosomes or lysed synaptosomes just prior to binding experiments using the technique described by Bradford (21).

Toxin probe preparation. Natural toxins were used as obtained, purifed from cultures Synthetic tritiated PbTx-3 and unlabeled PbTx-3 were prepared by chemical reduction of PbTx-2 using sodium borotritiide or sodium borohydride,

respectively. Toxin PbTx-7 was produced by identical chemical reduction of PbTx-1 using borohydride. Precursor toxins were mixed with equimolar reductant, each present in saturated solution. Under stirring conditions, the reactants were mixed and allowed to react for 3.5 min, after which time excess acetone was added as sacrificial substrate (reduced to propanol). The solvent and propanol was evaporated, and the residue was redissolved in minimal acetone. Acetone-soluble material was thin-layer chromatographed on silica gel plates using ethyl acetate/petroleum ether 70/30 as solvent, followed by high pressure liquid chromatography using an isocratic elution (1.4 mL/min) solvent of 85% methanol/15% water and monitoring absorbance at 215 nm.

Tritiated toxin was quantified employing uv HPLC detector tracings and standard curves were developed using unlabeled toxin PbTx-3. Radioactivity was determined using liquid scintillation techniques and appropriate quenched tritium standards. HPLC-purified radioactive PbTx-3 has a specific activity of 10-15 Ci.mmole, or one-fourth the specific activity of the chemical reductant. Aliquots of tritiated toxin are stored under nitrogen atmosphere at -20°C in theyl alcohol solution. Labeled toxin is stable for 4-6 months, repurification by HPLC being performed as necessary.

Other toxins. Other brevetoxins were used as purified from cultures. Potency of individual brevetoxins was measured using Gambusia fish bioassay (2,5).

Binding assays. Binding of tritiated toxin was measured using a rapid centrifugation technique (9). Binding assays were performed in a binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/mL bovine serum albumin, and 0.01% Emulphor EL-620 as an emulsifier; the latter being necessary to solubilize the high concentration of unlabeled PbTx-3 used in measurement of nonspecific binding. Binding experiments were also conducted in a depolarizing medium consisting of 135 mM KCl, 5.5 mM glucose, 0.8 mM magnesium sulfate, 1 mg/mL bovine serum albumin in 50 mM HEPES (pH 7.4). Synaptosomes (40-80 ug total protein), suspended in 0.1 mL binding medium minus BSA were added to a reaction mixture containing tritiated PbTx-3 and other effectors in 0.9 mL binding medium in 1.5 mL polypropylene microcentrifuge tubes. After mixing and incubating at 4°C for 1 hour, samples were centrifuged at 15000 g for 2 minutes. Supernatant solutions were aspirated and the pellets were rapidly washed with several drops of a wash medium (9). The pellets were then transferred to liquid scintillation minivials containing 3 mL scintillant and the bound radioactivity was estimated using liquid scintillation techniques. Nonspecific binding was measured in the presence of a saturating concentration of unlabeled PbTx-3 (10 uM) and was subtracted from total binding to yield specific binding. Free tritiated probe was determined by counting directly an aliquot of the supernatant solutions prior to aspiration.

D. Synthesis of Photoaffinity Probes

Two separate photoaffinity probes have been synthesized. Each probe has inherent in it advantages and disadvantages. Our original rationale was to synthesize toxin-linked organic azides which exhibited equivalent dissociation constants to the intact toxins, and which could be radio-synthesized with high specific activity. The photoaffinity probes should also be stable, both in reagent form and once covalently-linked to the specific binding site.

Following synthesis, which will be described below, we undertook a series of competitive displacement experiments in rat brain synaptosomes using unlabeled photoaffinity probes to evaluate the binding equilibria of the probes. This was done several times, the first time immediately following synthesis and purification, and the last time several months after synthesis to evaluate stability.

PbTx-3 Para-azidobenzoyl Ester. 13.7 grams of p-aminobenzoic acid (0.1 mole) was dissolved in a warm (40°-50°C) solution of 18 mL concentrated sulfuric acid in 100 mL water; the resulting solution was cooled in an ice bath. The amine was diazotized with a solution of 8.25 g sodium nitrate in 75 mL distilled water, the excess nitrous acid was removed with urea and 1.5 g powdered charcoal was added to a stirring solution. The charcoal was filtered after 1 hour, and a solution of sodium azide (10.8 g) in 60 mL of water was added dropwise to the cold, stirred filtrate. Nitrogen was immediately evolved, the solution became turbid, and a light tan precipitate formed rapidly. The suspension was cooled in an ice bath for one hour, and was then placed in a refrigerator overnight. The solid was then filtered, washed with a 10% sodium carbonate solution and then water, and was dried in a vacuum dessicator to constant weight (MP 184°-185°C, literature 185°C). An initial coupling reaction was attempted (unsuccessfully) which involved derivatizing the p-azidobenzoic acid with thionyl chloride to form the p-azidobenzoyl chloride derivative (Figure 3). This derivative was then mixed in equimolar amounts with PbTx-3 in pyridine solution and allowed to react overnight at room temperature with stirring. We were not able to demonstrate any ester formation using this scheme and it was abandoned in favor of the next technique. Ten mg p-azidobenzoic acid was dissolved in 0.23 mL tetrohydrofuran and was added to a solution of 4.630 mg PbTx-3 in 1.0 mL THF. The mixture was heated for 1 hr at 60-70°C in a sealed tube in an oil bath. Solvent was then evaporated with a stream of nitrogen and the mixture was thin-layer chromatographed on silica gel 500 u plates using 80/20 ethyl acetate/petroleum ether as solvent system. Standards of both PbTx-3 and p-azidobenzoic acid were run in parallel chromatographs. The coupled photoaffinity probe-toxin was visualized with iodine vapor (not uv light) and the appropriate band was scraped and eluted with methanol. The purified photoaffinity probe was stored at -20°C in methanol solution in the dark (Figure 4).

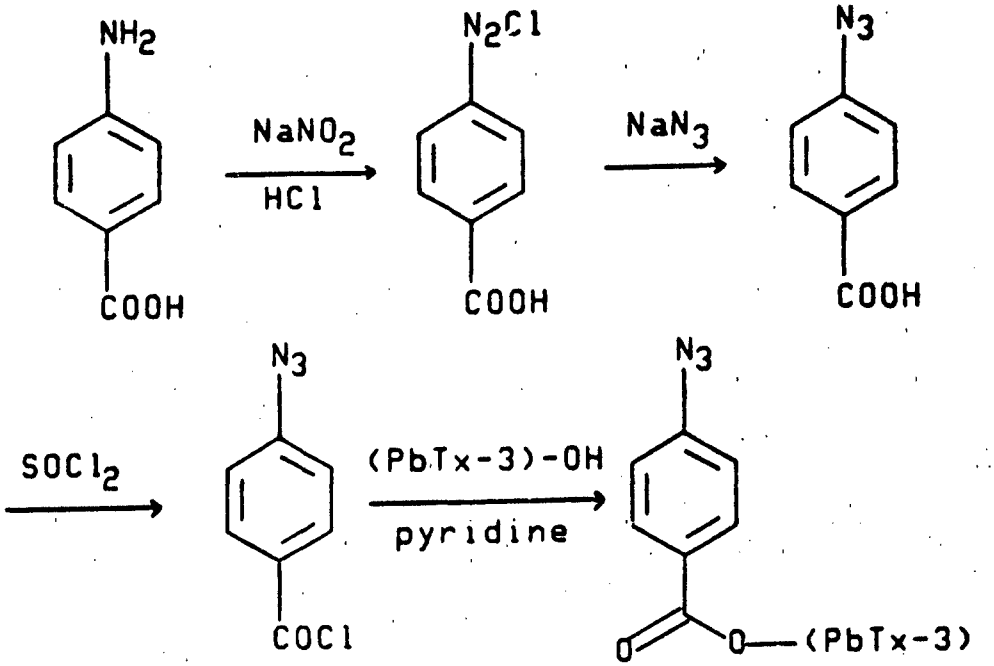


Figure 3. Synthesis of PbTx-3-p-azidobenzoyl ester. Thionyl chloride intermediate.

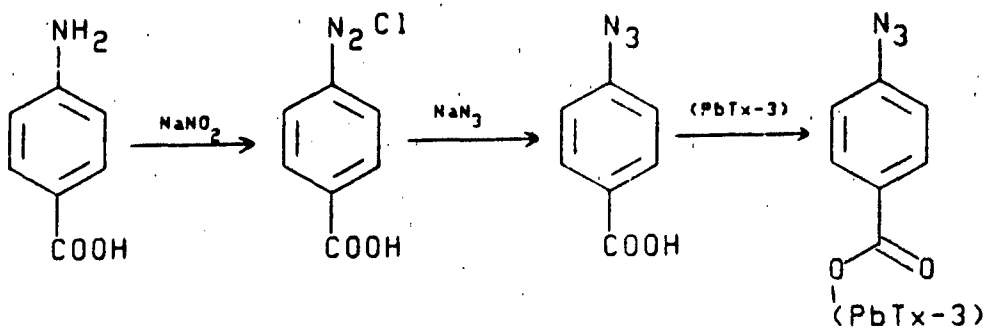


Figure 4. Synthesis of PbTx-3-p-azidobenzoyl ester. Direct esterification.

The PbTx-3-p-azidobenzoyl ester photoaffinity probe produced as a result of the scheme outlined in Figure 4 is labeled with tritium by utilizing tritiated PbTx-3 probe, and thus its theoretical maximum specific activity is about 15 Ci/mmole. The photoaffinity probe synthesis described below has the potential for yielding specific activities in excess of several hundred Ci/mmole by iodination ortho to the phenolic functionality.

Synthesis of 2-p-hydroxyphenyl-3-p-azidophenyl propionic acid and derivatives. This photoaffinity probe is multipurpose in that its various derivatives I-III (Figure 5) enable a user to couple a radioiodinatable photoprobe to (i) amines, (ii) carboxylic acids, and (iii) alcoholic hydroxy groups. For the second and third type of application, some modifications of the core compound---2-p-hydroxyphenyl-3-p-azidophenyl propionic acid---are required. The core compound itself is suitable for direct coupling with amines (I). The ethylenediamine monoamide (II) (Figure 6) allows for coupling to carboxylic acids, and is the technique utilized for PbTx-3-succinate coupling. Finally, the O-tetrahydropyranyl derivative (III) of the phenolic moiety (I)(its synthesis will not be described herein as it does not bear on the subject toxins) can be esterified with alcoholic hydroxy groups by carbonyldiimidazole coupling, followed by deprotection with mild acid treatment.

p-acetoxyphenylacetic acid. p-Hydroxyphenylacetic acid (10g) was dissolved in 72 mL acetic anhydride. The reaction was started by the addition of 2-3 drops of conc. sulfuric acid and manual stirring with a glass rod. On cooling, the acetate separates, is filtered, dissolved in ether, washed 4 times with water, and is dried over P_2O_5 (yield 10.5 g, 82.3% theoretical).

2-p-acetoxyphenyl-p-nitro-cinnamic acid. Using 100g of the previous product, the semi-solid was poured on ice, extracted with ether, and the solvent layer was washed twice with ice water. The solvent was dried with sodium sulfate, and was then filtered and flash-evaporated; the residue was used without further purification. To 100g p-nitrobenzaldyde, 182 mL acetic anhydride and 91 mL triethylamine were added and the solution refluxed for 2.5 hour. The resulting dark liquid was poured on an ice-water mixture acidified with sulfuric acid and was stirred in the cold. An oil separates which solidifies in one-half hour. It was filtered by suction, washed thoroughly with ice water; and was recrystallized from boiling acetic acid (500 mL), and 10 mL acetic anhydride (yield 138.5 g, 67.8%).

3-p-amino-phenyl 2-p-hydroxyphenyl propionic acid hydrochloride. Fifty grams of the above product was hydrogenated in THF solution in a Parr hydrogenator with 10% Pd-charcoal. The solution was heated to prevent clogging of the gas inlet tube during reduction. After the theoretical amount of hydrogen was taken up, the warm solution was filtered by suction and the filtrate flash evaporated. The residue was refluxed for 1.25 hours in 700 mL 1:1 aqueous HCl

solution. After partial cooling, it was filtered on fluted filter paper, and was then allowed to crystallize in the refrigerator. The product was filtered on a glass membrane filter and was dried over KOH flakes in vacuum (yield 36 g; 81.1%).

3-p-azidophenyl 2-p-hydroxyphenyl propionic acid (Figure 5, I). The above product (36.7 g) was dissolved in a mixture of 22.5 mL sulfuric acid and 125 mL water. On cooling, a solid separates. $NaNO_2$ (10.5g) in 95 mL water was added dropwise to the cold stirred suspension. The azido compound precipitates. After 1 hour standing in ice, the product is filtered by suction and washed with cold water and is dried over KOH under vacuum. The dried product was recrystallized from 100 mL boiling glacial acetic acid, the precipitate filtered by suction, washed with cold acetic acid and dried over KOH in vacuum (yield 27.8 g; 78.6%).

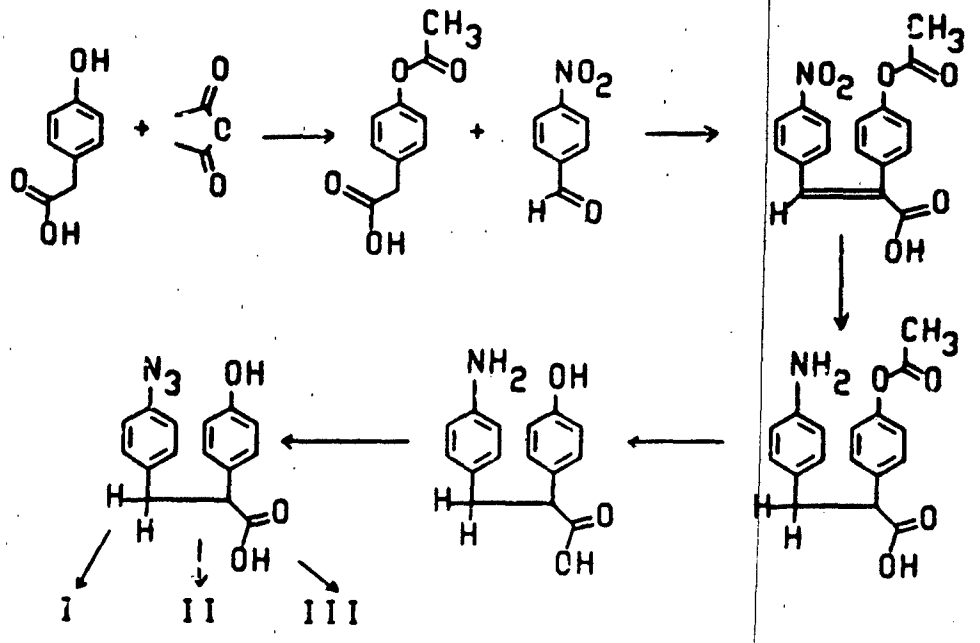


Figure 5. Synthesis of 3-p-azidophenyl 2 p-hydroxyphenyl propionic acid.

2-p-hydroxyphenyl 3-p-azidophenyl propionyl ethylene diamine monoamide (II)(Figure 6). 27.4g of (I) and 22 g of p-nitrophenol were dissolved in 40 mL dimethylformamide. Dicyclohexyl carbodiimide (16.2 g) in 10 mL DMF was added to the stirred solution dropwise in an ice bath. After addition, stirring was continued for 2 hours in ice and one hour after at room temperature, followed by addition of 10 mL glacial acetic acid and finally stirred 15 minutes longer. The urea formed was filtered off, and washed with 35 mL tetrohydrofuran. The filtrate was poured on ice (600 mL total volume) and an oil separates. The entire two-phase system was extracted with benzene, the solvent layer was washed 4 times with a saturated NaCl solution, and the solvent was dried using solid sodium sulfate. Twenty mL ethylene diamine was mixed with 100 mL benzene and was cooled on ice, and the previous solution was added to it dropwise under vigorous stirring over a period of two hours. The yellow precipitate was filtered by suction, the solid resuspended in 10% v/v sulfuric acid, and extracted with ethyl acetate to remove p-mitrophenol from the yellow gummy layer. A minimum volume of acid was used to achieve solubilization. The dark aqueous layer forms crystals upon standing in the cold. This is the sulfate salt of (II) (yield based on (I) is 51.6%). The salt is poorly soluble in water when compared with the hydrochloride. Preparation of the free base can best be accomplished by addition of sodium carbonate solution to the suspension until proper alkalinity is achieved. The free base separates and after drying can be recrystallized from hot absolute ethanol.

PbTx-3 derivative. PbTx-3 succinate is produced as we have previously described (18) using succinic anhydride in pyridine. PbTx-3 succinate (6.02 mg) was dissolved in 1 mL pyridine, mixed with two fold molar excess of DCC in 0.5 mL pyridine, and solid equimolar (II). The mixture was sealed in a screw-teflon capped tube and was heated for two hours at 85^{o}C in a Nujol bath. Following reaction, the bath was cooled, the pyridine evaporated under vacuum, redissolved in methanol and subjected to tlc in 80/20 ethyl acetate/petroleum ether. The PbTx-3-linked photoaffinity compound was visualized on the plate with iodine vapor, was scraped and eluted with methanol. The probe was stored at -20^{o}C in methanol solution.

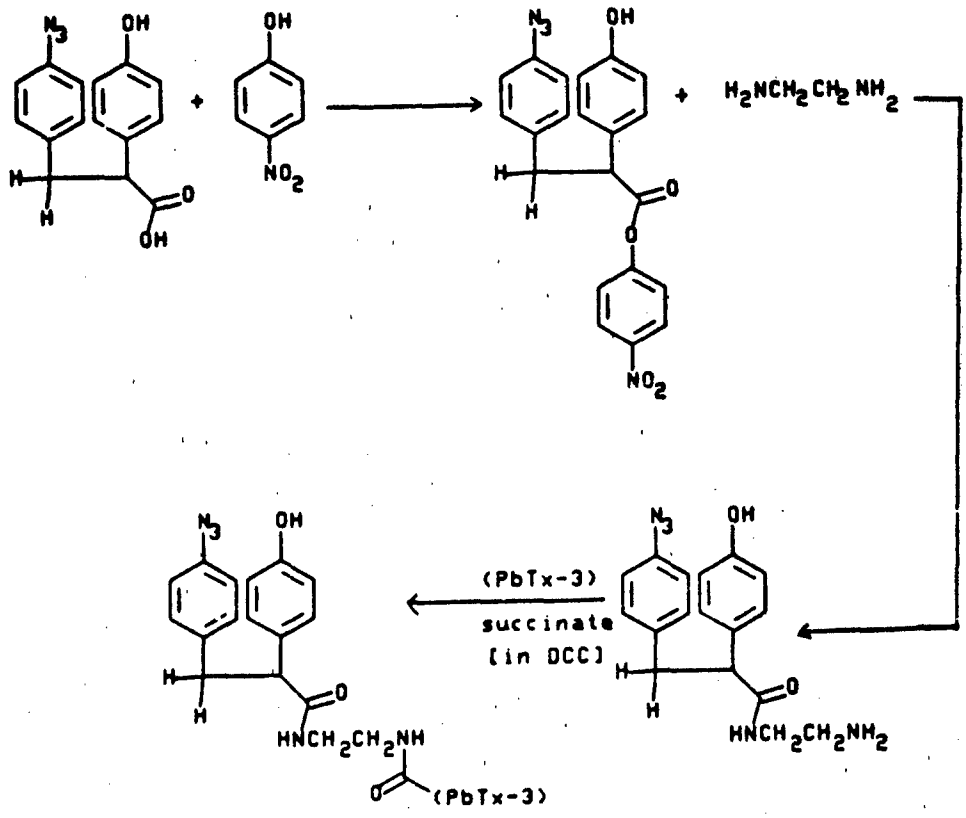


Figure 6. Synthesis of PbTx-3 linked 2-p-hydroxyphenyl 3-p-azidophenyl propionyl ethylene diamine monoamide (II).

V. Results and Discussion

A. Toxins

Number. We routinely isolate six brevetoxins from laboratory cultures of P. brevis, all based on the two polyether backbones (11). In logarithmic cells, the two predominant toxins are PbTx-1 and PbTx-2 (see Figure 1). In stationary cells, approximately the same relative amounts of PbTx-1 and PbTx-2 are present on a per cell basis, but now in addition PbTx-3, PbTx-5, PbTx-6 (based on the backbone present in PbTx-2), and PbTx-7 (based on the backbone present in PbTx-1) appear.

Abundance. In logarithmic phase cells, the respective yields of PbTx-1 and PbTx-2 are: 1.7 and 8.7 pg/cell (based on 80 liter extractions, n=10). In stationary cells, none of the "new" toxins exceed 5% of the total mass of toxin present (based on 80 liter extractions, n=3)(refer back to Table 2 for specific yields).

Effects of Culture Conditions. As we outlined in last year's annual report, it appears that culture stage plays an important role in the multiplicity of brevetoxins present. Our results, based intuitively on roughly 2400 liters of extracted culture (taken 80 liters at a time), and empirically on 1040 liters of extract, indicate that logarithmic cells contain primarily the two alpha-beta unsaturated aldehyde toxins; while stationary cells contain the multiplicity of toxins. We had originally thought that perhaps Ca^{2+} ions concentration played a role in toxin profile (based on a mistaken recipe for NH-15 medium preparation which was carried for six months), but subsequent examination of toxin production with respect to Ca^{2+} concentration (restoring concentrations to their higher value) indicates no shift. That we observe a greater number of toxins than we did when cultures were at the Medical School facility is unexplained, and we believe there is no way to examine the difference.

Hypothesis of Toxin Synthesis. Consistent with our observations on toxin profile and culture growth phase is the hypothesis that the toxins extracted from P. brevis may not be synthesized by the dinoflagellate as a normal cellular metabolite. This is, we feel, a rather revolutionary statement, that P. brevis does not synthesis toxin as a metabolic strategy or for any competitive advantage.

Consider for a moment that, as an example, a plastid or plasmid were responsible for the critical "metabolic machinery" necessary to produce brevetoxin from normal cellular substituents. Further consider that the toxins are in themselves deleterious to the dinoflagellates. Assuming that toxin synthesis is constituitive and not an inducible phenomenon, it is possible that a subletha' toxin concentration per cell can be maintained by vegetative binary fission as occurs during logarithmic phase. Upon changes in growth characteristics of cultures (as occurs in stationary

growth phase), however, that delicate balance is upset in favor of toxin synthesis.

Aside from excretion of the two aldehyde toxins PbTx-1 and PbTx-2 (which have been shown by a number of investigators to be intracellular in log phase), there are relatively few ways to detoxify these materials. We postulate that the dinoflagellate detoxifies the two alpha-beta unsaturated brevetoxins to the other toxins within the profile. This is done by normal detoxification reactions including aldehyde reduction (to PbTx-3 and PbTx-7), epoxidation (to PbTx-6), and O-acetate derivatization of the C-37 alcohol in PbTx-2 (to PbTx-5).

Not only is this idea plausible to invoke for P. brevis toxin profiles, but the idea is also consistent with Gonyaulax toxin profiles; i.e. that saxitoxin is synthesized using "machinery" which is partly plastid or plasmid encoded. The derivatives which result and which contribute to profile multiplicity include neosaxitoxin (N-hydroxylation), gonyautoxins 1-4 (sulfate derivatives of hydroxyls), gonyautoxins 5-6 (carbamoyl-N-sulfated derivatives), and epigonyautoxin 8 and gonyautoxin 8 and C3 and C4 (which are combinations of two detoxification pathways).

A step further removed is the situation with ciguatoxin production by Gambierdiscus toxicus, a dinoflagellate which produces ciguatoxin in the wild, but under controlled laboratory conditions does not. Perhaps G. toxicus does not cease ciguatoxin production in the lab, but rather merely becomes more efficient at "detoxifying" the potent material.

We believe it is important to note that in all cases the derivatized materials are less potent than are the parent molecules; and are also more water soluble in a general sense. Not only does this change in intuitive point of view shed light on the toxin-dinoflagellate relationship and the dinoflagellate's capability for dealing with these materials, but it also provides potential for further work on transfer of toxin synthetic capability.

B. Moleclar Pharmacology

Competitive Displacement of Tritiated PbTx-3 by Natural Brevetoxins. We have previously shown that PbTx-3 binds to site 5 associated with voltage-sensitive sodium channels, have determined a K_D of 2.9 nM and a B_{max} of approximately 7 picomoles/mg synaptosomal protein (see Figure 2). We also demonstrated in our last annual report that tritiated PbTx-3 could be displaced in a specific manner from its binding site by PbTx-2, or PbTx-3 (either natural or synthetic), but not by oxidized PbTx-2. Our initial observation was that displacement efficiency was linked in a positive fashion with potency in animals.

The sensitivity and specificity of the synaptosomal assay for site 5 using brevetoxin PbTx-3 is equivalent to the case for synaptosomal assay for site 1 using saxitoxin (Figure 7).

Using the additional natural brevetoxins we have developed specific displacement curves which correlate well with the potency of each individual purified toxin (Figure 8). We found it was very important to include the Emulphor EL-620 in all experimental tubes. The reason for this requirement, we surmise, is because of the differential lipid solubility of each of the natural brevetoxins and their tendency to form micelles.

In addition to developing displacement curves for the six toxins (n=2), we had sufficient toxin material for PbTx-1,-2,-3, and -7 to calculate K_is. These are shown in Figure 9. The results of Figures 8 and 9 are correlated in tabular form in a later section.

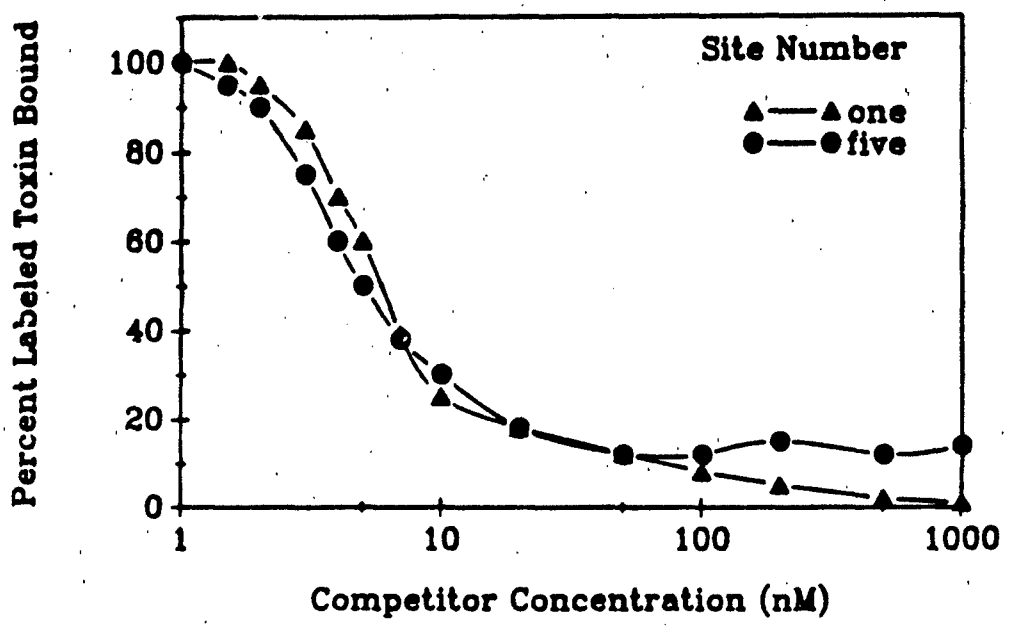


Figure 7. Specific Displacement of Labeled Toxins by Unlabeled Competitors, Sodium Channel Receptors. Site 1 probe is 10 nM tritiated saxitoxin; site 5 probe is 10 nM brevetoxin PbTx-3. Competitors are unlabeled saxitoxin and brevetoxin, respectively.

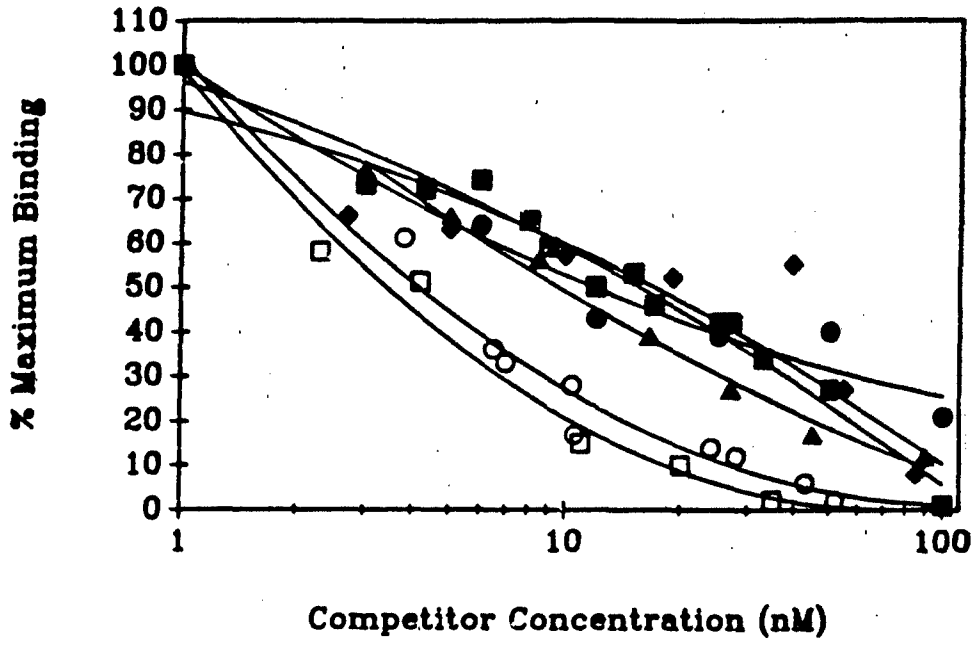


Figure 8. Specific Displacement of Tritiated PbTx-3 by Unlabeled Brevetoxins. Incubations, in the presence of 50 ug synaptosomal protein and 16 nM tritiated PbTx-3 (10.15 Ci/mmole) with increasing amounts of unlabeled PbTx-1(□), PbTx-2 (■), PbTx-3 (●), PbTx-5 (▲), PbTx-6 (◆) or PbTx-7 (○), were for 1 hour at 4°C. Each point represents the mean of three triplicates. The ED_{50} in each curve is given in Table 3.

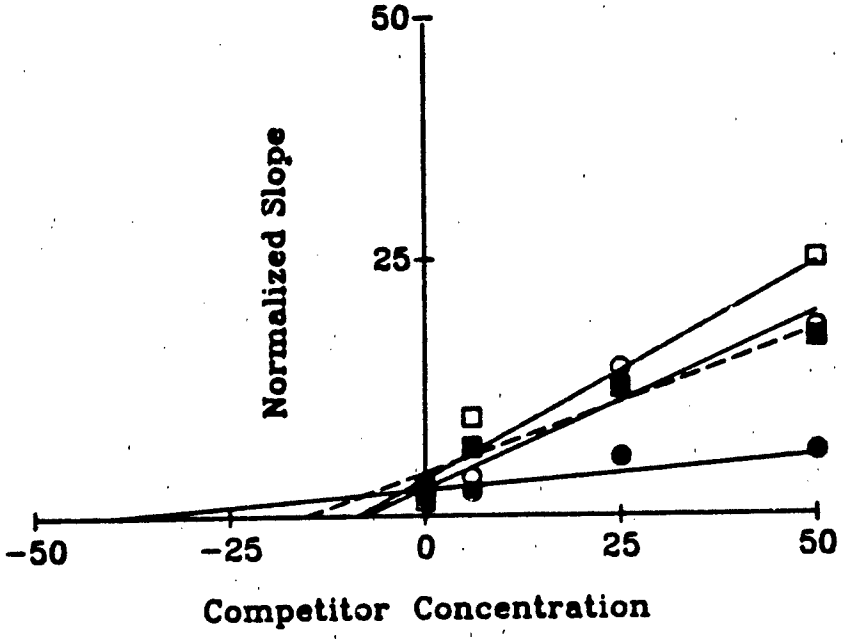


Figure 9. K_i Determinations for Brevetoxins. Rat brain synaptosomes were incubated with tritiated PbTx-3 concentrations of 2,5,10 and 20 nM, and unlabeled PbTx-1 (□), PbTx-2 (■), PbTx-3 (●), or PbTx-7 (○) at concentrations of 5, 25, 50, and 100 nM. Double reciprocal Lineweaver-Burke type plots were developed for each toxin and indicated competitive inhibition of binding. The slopes of the individual lines wer plotted against the competitor concentration in each case. K_is in each case are determined by the intersection of each line with the competitor axis, and is equal to $-K_i$.

We have collected sufficient competitive displacement information on PbTx-1,-2,-3,and-7 only, principally because they are abundant enough to gather sufficient toxin for the large number of individual tubes required for each experiment. Each line in Figure 9 is the result of at least 36 individual measurements. The K_is determined thus far approximate relative potencies of the materials; i.e. those with lower K_is are more potent. This work is still in progress however and additional replications are required before we prepare the results for juried journal publication. In addition, we plan to continue collecting PbTx-5 and -6 for use in similar experiments.

Competitive Displacement of Tritiated PbTx-3 by Photoaffinity Labels. This series of experiments was undertaken with two main goals. First, it was important to determine if, once the toxins were covalently linked to each photoaffinity molecule, each complete photoaffinity probe was capable of interacting with the specific site associated with voltage-dependent sodium channels---and also to determine in an approximate fashion what the affinity was for the site. The second investigation was undertaken to obtain some idea of the stability of each of the probes when stored. It should be noted that each of the prepared probes are non-radioactive and we expect we shall have to repeat stability experiments once radioactive probes are synthesized.

Both experiments are represented as a single graph, Figure 10.

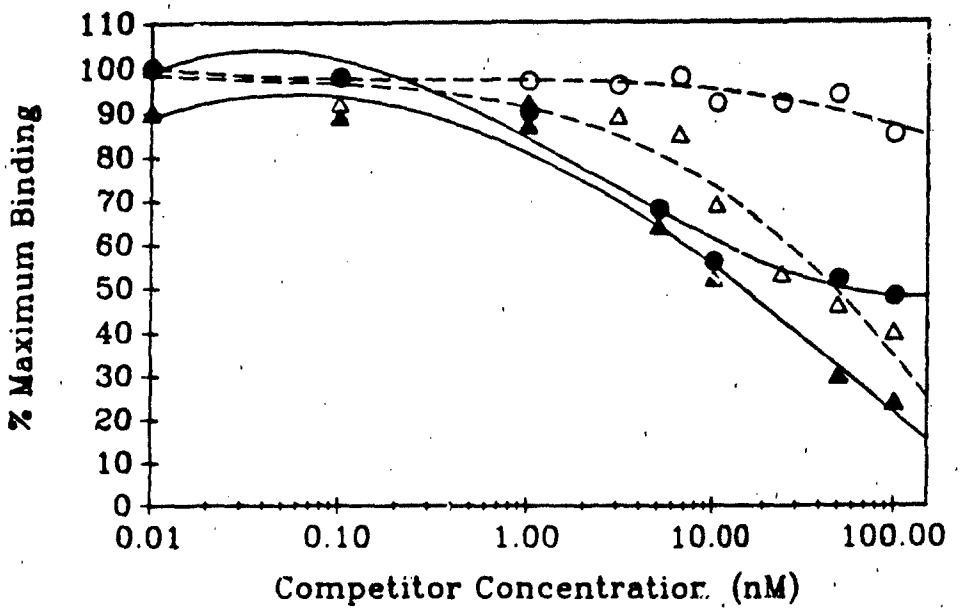


Figure 10. Competitive Displacement of Tritiated PbTx-3 by Photoaffinity Probes. (△▲) PbTx-3-p-azidobenzoyl ester; (○●) PbTx-3-2-p-hydroxyphenyl-3-p-azidophenyl propionic acid derivative. Open figures are using the same preparations, but carried out after 3 months of storage at -20°C in ethanol or methanol solution.

As can be seen from the curves in the figure above, the smaller azidobenzoyl ester derivative is slightly more effective at displacing tritiated PbTx-3 from its specific binding site (ED_{50} approximately 18 nM) than is the propionic acid derivative (ED_{50} approximately 50 nM). The former derivative also appears to be more stable, its ED_{50} being about 60 nM after 3 months storage---and the latter

derivative is almost totally degraded by this time. It is important to note, however, that each photoaffinity-derivatized toxin probe is still able to interact with specific site 5 associated with the channel, and therefore the potential for covalently-linking the site to a radioactive probe is high. These studies are just beginning.

TABLE 3. COMPARISON OF ED_{50}, K_i, AND LD_{50} FOR SYNAPTOSOME AND FISH BIOASSAYS

Toxin	Synaptosome ED_{50} (nM)	Synaptosome K_i (nM)	LD_{50} (nM)
PbTx-1	3.5	7.1	4.4
PbTx-7	4.1	8.9	4.9
PbTx-2	17.0	16.1	21.8
PbTx-3	12.0	37.0	10.9
PbTx-5	13.0	----	42.5
PbTx-6	32.0	----	35.0

ED_{50} are defined as the toxin conc at which 50% displacement of tritiated PbTx-3 from sodium channels occurs. LD_{50} are determined by incubation of Gambusia affinis with toxin in 20 mL seawater for 60 minutes. K_i are determined as described in the text and should be considered preliminary data.

In the synaptosomal assays (Figures 8-9), the displacement curves for brevetoxins possessing PbTx-2 type structural backbones show 50% displacement at 10-30 nM competitor concentrations. By comparison, PbTx-1 and PbTx-7 displace tritiated PbTx-3 at much lower concentrations; in both cases 50% displacement occurs at about 4 nM competitor concentrations. T-test analysis revealed no significant difference between the PbTx-2 backbone type toxin ED_{50}s ($p<0.01$), or between PbTx-1 and PbTx-7 ED_{50}s ($p<0.01$), but statistically significant differences were found between the curves generated by the two backbone classes.

The comparison of Gambusia affinis fish bioassays shown in Table 3 with the ED_{50}s and preliminary K_i data for each respective assay indicates that the two more potent ichthyotoxins, PbTx-1 and PbTx-7, are most efficient on a molar basis in displacing labeled PbTx-3 from its specific site of action in synaptosomes.

The affinities of the toxins in the synaptosomal assays theoretically are based on differential structural considerations involved in the binding to the site on the sodium channels as well as lipid solubilities of each of the materials. It is noteworthy that PbTx-1 and -7, the two most hydrophobic toxins, are also the most potent and bind with tightest affinity to the site of action. The greater binding affinity of these two toxins may be a function of their flexibility across the D,E,F,G polyether portion (which confers about a 40^{o} bending capability) as opposed to the rigid character of PbTx-2-like toxins. This added flexibility may allow these two toxins to conform better to the topography of the brevetoxins binding site.

It has been hypothesized that the brevetoxin binding site lies in an hydrophobic portion of the channel (8). This is consistent with the potency and hydrophobicity of PbTx-1 and -7. It is also noteworthy that oxidized PbTx-2 is impotent in fish bioassay, and that it lacks any capability to displace tritiated PbTx-3 from the binding site (9). It is our contention that the substituent character on C-42 in part determines solubility (and hence access to its synaptosomal site of action), and that the distal end of each backbone type carries the active portion of each toxin. Additional brevetoxin derivatives are being synthesized to test this hypothesis.

VI. Conclusions

Laboratory cultures of Ptychodiscus brevis produce at least six different polyether toxins, derived from one of two structural classes. During logarithmic phase, two of the toxins predominate and both are alpha-beta unsaturated aldehydes based on different structural backbones. Upon reaching stationary phase, four additional toxins appear, three based on the structural backbone present in PbTx-2, and an additonal single toxin based on the structural backbone present in PbTx-1. The additional toxins have been hypothesized to arise from classical detoxification reactions carried out on the two alpha-beta unsaturated aldehyde toxins.

Each toxin displaces tritiated PbTx-3 from its specific site of action; all ED_{50}s occur in the 3-30 nM concentration ranges with the more lipid soluble toxins being more efficient at displacing labeled toxin. The ability to displace radioactive toxin from the binding site correlates in a positive fashion with fish potency. Preliminary K_i calculations for four of the toxins indicate a similar positive correlation.

Two photoaffinity labels have been synthesized using PbTx-3 toxin. Each possesses the capability of displacing tritiated PbTx-3 from its specific site of action, indicating the active site still recognizes these derivative toxins. ED_{50} values fall in the 18-50 nM concentration range.

VII. Recommendations

Investigation of molecular binding pharmacology using the brevetoxins has progressed well during this past year. For this upcoming year, we plan to complete the following tasks:

(1) complete examination of tritiated PbTx-3 binding under potassium ion depolarizing conditions. We have thus far been unsuccessful in detecting any difference between normal polarized state and under conditions of membrane depolarization;

(2) fully explore synthesis of radioactive brevetoxin PbTx-3 photoaffinity labels and begin initial characterization of the brevetoxin binding site upon covalent binding;

(3) examine the binding of tritiated PbTx-7 (much more potent than PbTx-3 and should bind tighter) to rat brain synaptosomes. This investigation will be correlated with PbTx-3 binding to give us the first data on binding of the newer structural backbone. The tritium probe PbTx-7 is available;

(4) if (3) is successful, examine competitive displacement of tritiated PbTx-7 using natural brevetoxins;

(5) attempt to produce a photoaffinity probe using the newer structural backbone (covalently attached PbTx-7). This material should bind tighter than the probes synthesized using the PbTx-3 backbone.

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